Development and Maturation of the Immune System: Vulnerability to Toxicants

The take-home message I would like to leave about developmental immunotoxicity is, from my experience, that the developing immune system is highly sensitive. In some cases markedly more so than the adult immune system. And I think this statement is probably going to expand as we learn more and more about developmental immunotoxicology. But this field is still in its infancy.

There is a decent literature base which we've tried to summarize in a report that should come out in the next couple of months in Environmental Health Perspectives (note: the paper mentioned was published in Environmental Health Perspectives 108 Supl. 3:463-473, 2000). Ralph Smialowicz of the U.S. EPA at Research Triangle Park, North Carolina, was a co-author. We've tried to summarize the information that's out there. Again, it's a highly sensitive system, and if I don't leave any other message that's one I would like to leave.

This is a scheme for development of the immune system.

Here we see the early pluripotent stem cells that under local microenvironmental influences will produce all of the white blood cells that comprise the immune system. During development the immune system is largely established; ontogeny of this system is largely a pre- and early-perinatal event, which I think is part of why the system is so sensitive to chemical or other exposures.

The system is characterized by carefully timed and highly regulated waves of proliferation and differentiation that are very sensitive targets for some chemical exposures. We know that genetic abrogation can occur in part of this system -- for instance lymphocytes (T-cells and B-cells). The genetic loss of ability to produce those cells results in a syndrome called SCIDs (severe combined immune deficiency), and the postnatal consequences are critical to life-threatening. In like manner, certain chemicals will interfere with different parts of the establishment of the immune system.

Diethylstilbestrol (DES) is a very potent and selective targeter of the progenitor of the T-cell. Dioxin is also a very potent and selective targeter of the prothymocyte. With either of these chemicals, low level exposure results in impaired colonization of the thymus by these cells. We see tremendous thymic involution.

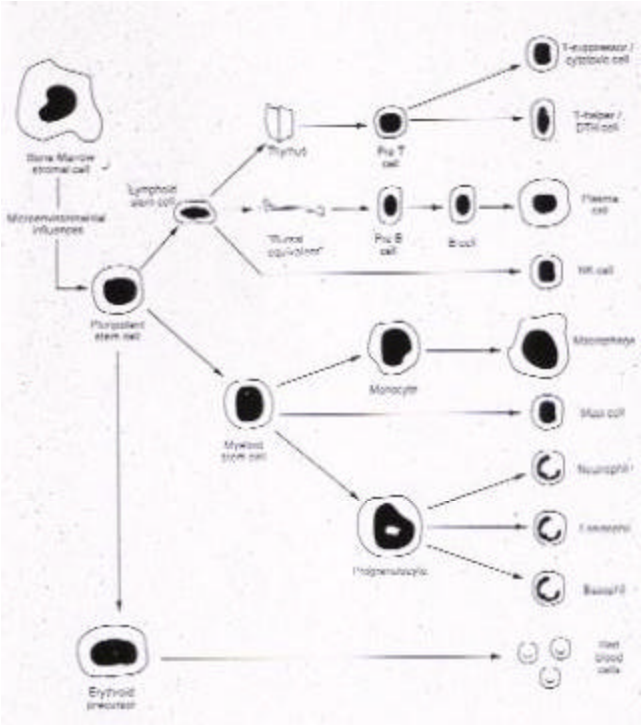
This system is very amenable to targeting by a number of chemical agents. I don't mean to imply that DES and TCDD only target the prothymocyte, however the latter seems to be a very sensitive cell. In our laboratory we isolated prothymocytes and evaluated estrogen receptor expression on these cells. They do have a relatively high-affinity estrogen receptor, which might explain their sensitivity to DES and might have implications for environmental estrogens as well, bisphenol and so forth that at low levels may affect development of the immune system.

In addition to being characterized by waves of proliferation and differentiation, this system is characterized by multiple shiftings of hematopoietic compartments during development. As early as 24 hours in some vertebrate species we have these cells of fetal

origin formed on the area vasculosa of the fetal yoke sac, and shortly thereafter they'll migrate to blood islets within the yoke sac. As the yoke sac diminishes they'll migrate to fetal liver. Fetal liver is the primary hematopoietic organ throughout most of gestation. It's similar to bone marrow in the postnatal arena. A number of chemical agents are very effective at targeting immune cells and fetal liver.

As the fetal liver becomes more devoted to metabolism these stem cells will migrate to bone marrow. This is the perinatal situation where our bone marrow becomes hematopoietic. Early in life these are the long bones of our legs and our arms. As we age these bones fill with yellow fat and become less devoted to hematopoiesis, and we have a final shifting of the primary hematopoietic compartment to the bones of our axillary skeleton: our ribs and our vertebra, our sternum and our pelvis. Thus, when adults donate bone marrow this is usually collected from the wings of the ileum (pelvis). So we have multiple migrations of these compartments, and these are all subject to chemical targeting.

This system is the one shown up here for humans. The developmental pattern in the mouse is similar, except that timing is quite different as to when the mouse produces these cells as compared to when the human does.

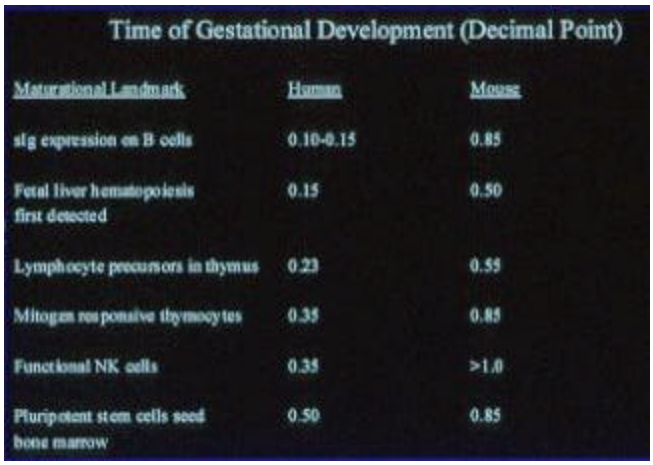


Ralph Smialowicz and I developed a comparison between humans and the mouse in terms of maturational immune system landmarks and when they occur. For instance, surface immunoglobin expression on B-cells in humans occurs at 10 to 15% of the way through gestation. You see again the mouse is quite different here, being 85% of the way through gestation before we see the same landmark in the mouse. Fetal liver hematopoieses is first detected in humans 15% of the way through gestation; in the mouse halfway through gestation.

T-cells have the ability to clonally expand 35% of the way through gestation, or at about the end of the first trimester in humans. And again, at 85% of the way through gestation in the mouse.

The message here is that ontogeny of the immune system, the basic system occurs earlier in humans than in the mouse. Most immunotoxicity studies expose animals in late gestation. In the mouse we're exposing them at a time when immune development is similar to that occurring in humans, which is quite early. We need to remember this when we try to make comparisons. You see functional natural killer cells here in humans, end of the first trimester, but functional NK cells don't show up until birth or thereafter in the mouse.

So while the overall system is laid down in a similar manner in mouse and humans the timing is quite different, and for purposes of risk assessment that can be quite important.



Time of Gestational Development (Decimal Point)

Maturational Landmark	Human	Mouse
sIg expression on B cells	0.10-0.15	0.85
Fetal liver hematopoiesis first detected	0.15	0.50
Lymphocyte precursors in thymus	0.23	0.55
Mitogen responsive thymocytes	0.35	0.85
Functional NK cells	0.35	>1.0
Pluripotent stem cells seed bone marrow	0.50	0.85

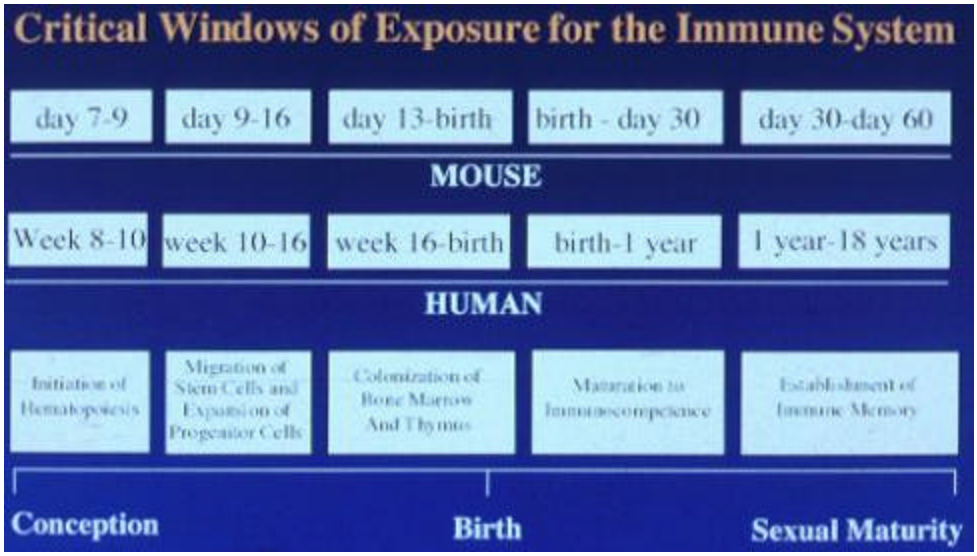
We were asked at a children's health workshop late last year in Richmond to try to produce critical windows of exposure, a topic applicable to this meeting as well. A panel of about a dozen of us decided we really couldn't do this, with chemicals at least. We estimated as best we could in the mouse model and in the human when critical exposure windows might be. One of these was initiation of hematopoiesis in which stem cells are activated to produce progenitors, which then clonally expand. This would seem likely to be a sensitive time, and we know that for some chemicals it is.

Similarly, at days 9 to 10, migration of stem cells and further expansion of progenitors occurs and may be a sensitive time. We speculated on other sensitive times; this figure's will be in a workshop overview paper coming out soon, so it will be readily available. [Note: the paper mentioned is now published, Environmental Health Perspectives 108, Suppl. 3:483-490, 2000]

The organizers of the meeting wanted us to put chemicals down here with arrows, for instance chlordane, and point up here to days 9 to 16, and say this is the sensitive time. Our decision was that there was not a sufficient database for any chemical to allow us to establish the most sensitive time in development.

Chlordane we know affects early events as well as late events. Beyond chlordane we've rarely considered earlier time periods when we're first establishing pluripotent stem cells,

and what effects chemicals might have on that process. We just don't have the answer to that.



I selected a few chemicals as examples of consequences of early exposure to immunotoxicants. These chemicals are chlordane, benzo[a]pyrene, diethylstilbestrol, and dioxin. I know a number of you are familiar with and have worked with these.

When I began my post-doctoral appointment as a toxicologist, I shared the lab with another post-doc named Benny Blaylock (an immunologist from Arkansas). Benny is head of the Toxicology Department now at University of Louisiana, Monroe. He did his dissertation work with chlordane. I remember him commenting, "It was just amazing to me that I exposed pregnant mice to low levels of chlordane that produced limited to nondetectable immune effects in adult mice, yet the offspring of these mice were immunologically different the rest of their lives." His dissertation work involved following these mice to 18 months of age, and showed significant immunosuppression in these now-geriatric mice. So again, at levels that have minimal to nondetectable immune effects in the adult animal the offspring were immunologically different as far out as we looked which was 18 months in this case.

Chlordane of course is a cyclodiene insecticide and is widely distributed. To illustrate this fact, we had a problem in our area recently with box turtles, of all animals, that were exhibiting -- these were wild-caught animals -- a vitamin A deficiency. I tend to get the reptile calls for the college and ended up evaluating these turtles. We live in a very pristine area, in the mountains in the western part of Virginia. To make a long story short, the turtles had considerable chlordane in their livers, which appears to have caused the vitamin deficiency. This was a fairly-widely distributed compound but its use in this country was banned in the late '70s. Nevertheless, there appears to be enough left in our area to cause health problems in wild turtles.

Benzo[a]pyrene is a polycyclic aromatic hydrocarbon and is produced by combustion of fossil fuels, among other things. It is a potent carcinogen, and one of the carcinogens of concern in cigarette smoke. It is a potent anti-proliferative agent and, therefore, an

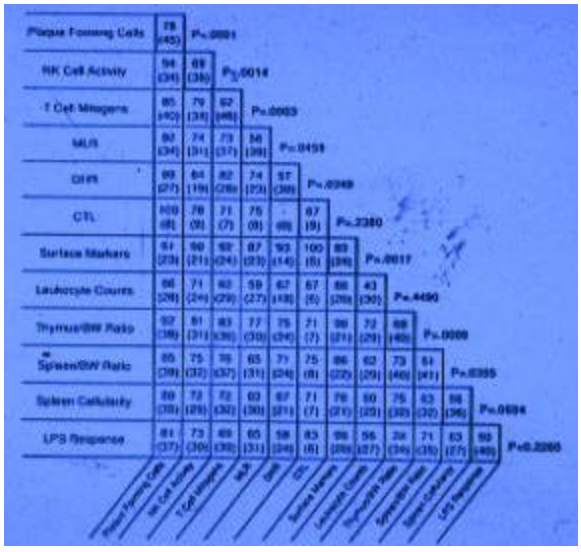
immunotoxicant, it also binds the Ah receptor, and likely has other immunotoxicity effects as a result of that.

Like chlordane, prenatal exposure to benzo[a]pyrene will produce permanent postnatal immunosuppression in mice. This is something we don't see in adults. Effects in the adult tend to be transient, yet in the prenatal arena we've observed changes that are permanent with that compound.

These two compounds on the right, diethylstilbestrol being a nonsteroidal estrogen, a model estrogen and the first demonstrated transplacental carcinogen, is also a potent immunotoxicant. Estrogens at physiological levels are immunomodulating , which in part explains superior immune responses to men. Women can mount a stronger T-cell immune response and produce higher levels of antibodies, higher titers. These are estrogen-driven traits. However, there's a price to be paid for this difference in females. Women are considerably more prone to develop autoimmune disease. For instance, systemic lupus occurs 11-to-1 in women over men. So this compound is immunomodulating at physiologic levels. But at pharmacologic levels this is immunotoxic. And at very low levels of exposure the same is true of dioxin.

These latter two compounds produce a very prolonged postnatal immunosuppression. A recent concern, and a new concern of the NIH (which sent out a related RFA not long ago) is that exposure to these compounds may induce or exacerbate autoimmune disease in the human population. Such disease appears to be increasing. The possibility that environmental chemicals (prenatal or postnatal exposure) may be related to expression of autoimmune disease has not received much attention by researchers.

We were also asked to address risk assessment, and how we might approach this in developmental immunotoxicology. I'll explain what is often done.



This figure is from the National Toxicology Program's immunotoxic chemical testing paradigm that was developed in the B6C3F1 mouse. Chris Portier is in the audience and had a big hand in developing this system.

This is what I and many others have used. These are simply immune assays along this line here. For instance, the top assay is a plaque-forming cell assay , which measures

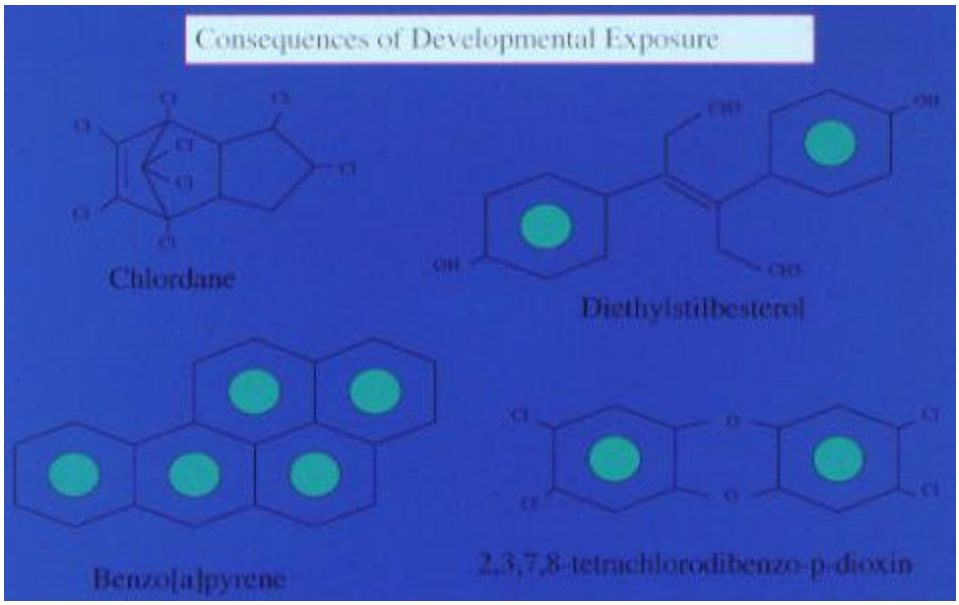
ability of the animal to produce antibody following challenge with a specific antigen. It turns out this is a very good test for detecting immunosuppression. If we have chemical-exposed mice and their ability to produce antibody is significantly below control levels, the predictive value for clinically significant immunosuppression is 0.78; there's about an 80% likelihood that this animal really is immunosuppressed. Basically, you don't want your ability to produce antibody to be depressed, according to this risk assessment paradigm.

By immunosuppressed I mean if we challenge the mouse with a bacterial pathogen (e.g., listeria) or with a syngeneic tumor cell, (the immune system plays a role in eliminating such cells), then we'll find that these animals cannot respond immunologically at a control level. So, that's what these predictive values are indicating.

This one is natural killer cell activity which has a 0.69 predictive value; 69%. For the ability of T-cells to clonally expand, 67%; leukocyte counts down here, 43% (not a real good test, not a high predictive value). You'll see the same assays are listed on this axis here (horizontal). That gives us a pairwise concordance for predicting immunosuppression. The plaque-forming cell assay paired with thymic weight, 92%, we're getting pretty high. And up here the cytotoxic T lymphocyte assay paired with the PFC assay, a hundred percent. This is the risk assessment paradigm I use.

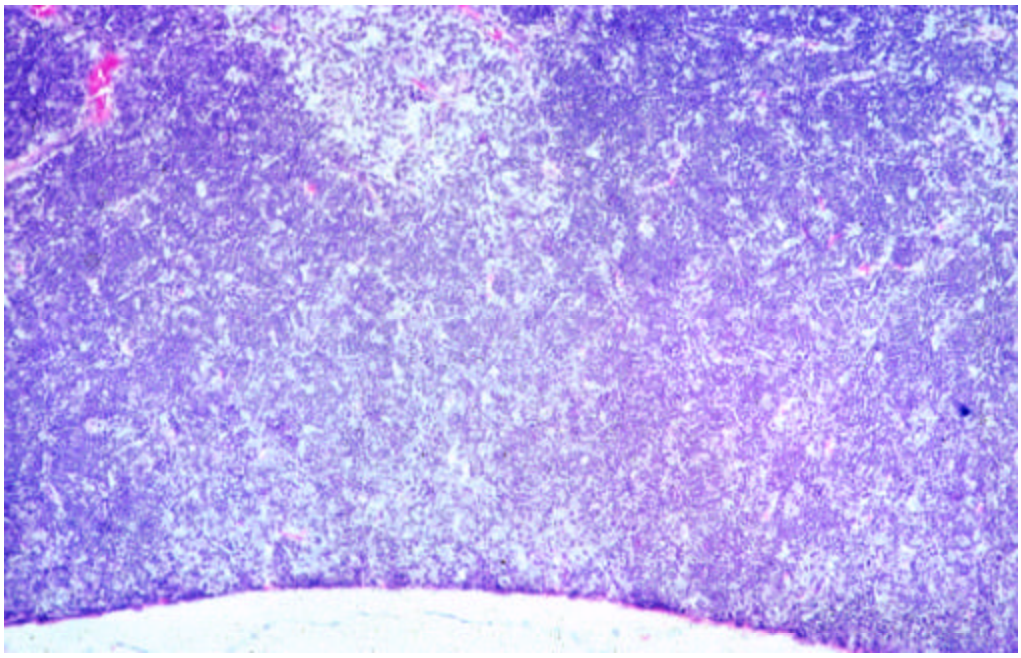
It's felt that this system probably applies reasonably well to other species beyond the B6C3F1 mouse it's been developed in. How well does it apply to gestational exposure? Is the ability to produce antibody as important if it's depressed following prenatal exposure? If I let these animals be born and evaluate them six weeks later does it mean the same thing? Is the predictive value the same as in the system that this paradigm was developed in? That's a question nobody knows the answer to.

It wouldn't be overly difficult to reproduce this paradigm in a developmental arena so that we would know what those predictive values are. And I think that would be a worthwhile thing to do for estimating damage to the immune system from early exposure rather than late-life exposure. And I would guess those predictive values would be fairly similar but, again, that's my estimation.



One of those immune tests on the previous slide was simply thymic-to-body-weight ratio, or thymic mass, thymic cellularity. This is the slide of a thymus of a control animal. There are two thymic lobes that are located in the caudal neck/cranial thoracic cavity area in the mouse. The magnification here is 50x.

The thymus is important because it contains developing T-cells, and if we give a chemical that diminishes thymic size we have fewer T-cells to participate in immune response and this might cause immunosuppression. Notice in this slide that the control thymic lobe is considerably bigger than the field of the slide.

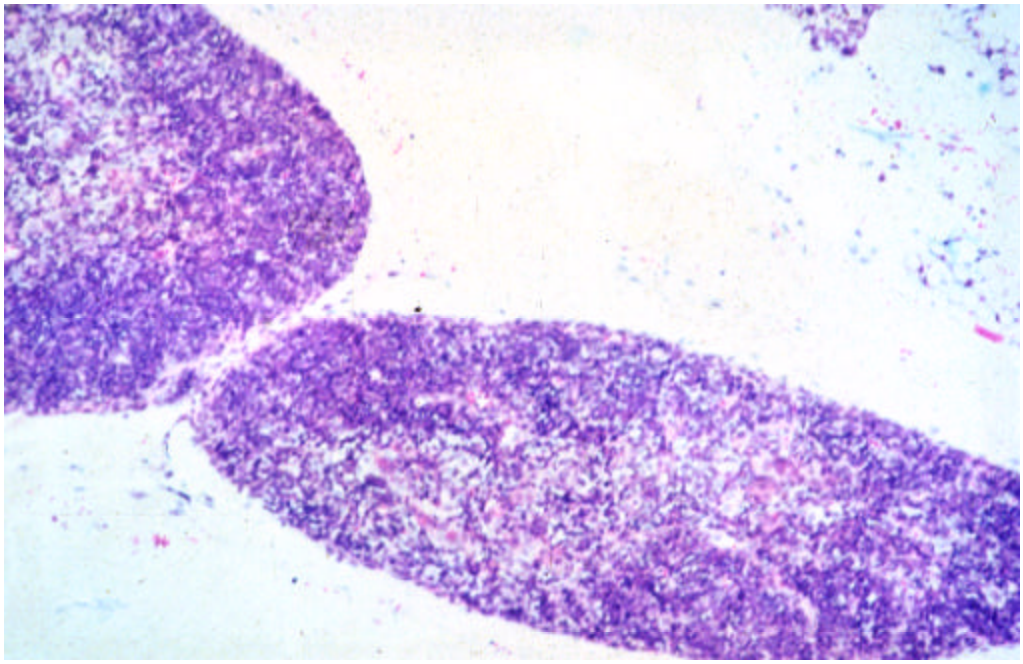


This slide is from an adult mouse that was exposed to eight milligrams per kilogram DES, a dose that is lower on a mg/kg basis than Dr. Rogan quoted for human females

given of a gram or more DES for terminating lactation. Males with prostate cancer receive high doses as well.

So let's see what happens to the thymus of this mouse. You see it's quite a bit smaller now, when exposed to eight milligrams per kilogram of DES. This one thymic lobe fits very readily on the field of this slide. I could drag the other one over and put them both on. So we see a very marked thymic involution with this pharmacologic exposure in a postnatal situation to DES. This was about a 12-week old C57Bl/6 mouse.

Does the same thing happen in development with DES? The answer to that question is, yes.



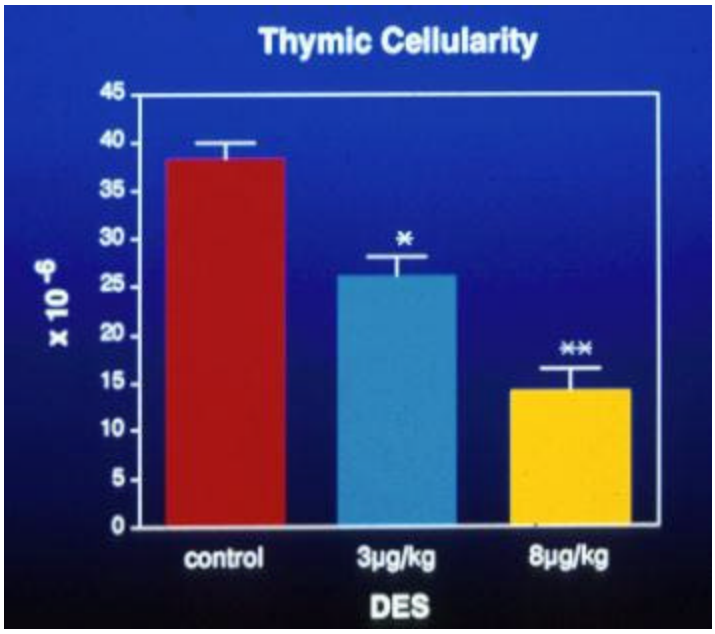
This graph shows thymic cellularity, the count of pre-T-cells in the thymus as an indicator of thymic size. The DES exposure was three or eight micrograms per kilogram. And you can see this early exposure to DES is produced an involution of the fetal thymus on day 18 of gestation when the thymus was taken. The point I wanted to make with this slide is, the adult exposure I showed you was eight milligrams per kilogram. In this case exposure to the pregnant animal was eight micrograms per kilogram, which is a thousand-fold less.

This exposure is by oral gavage, which means we've got first-pass effect in the liver removing a considerable amount of this DES. But I'm going to over-estimate that 5% of DES crosses the placenta and is available to the fetus.

In these mice we have a variable number of fetuses, but if we figure that this distributes to 10 fetuses and 5% crosses the placenta, then 5% is one-twentieth times 10 fetuses is one two-hundredth the maternal dose per fetus. We're already at one one-thousandth the level that the adult animal got that I showed you a moment ago. These fetal mice received approximately one two-hundred-thousandth of the dose (that's 200 times a thousand) that the adult animal received in the previous slides, and yet we see

comparable involution of the thymus. So, the system is very sensitive to early estrogen exposure.

Results have been comparable with dioxin. We have given maternal exposures that I would calculate picogram levels per fetus, yet we can show differences in the thymus with TCDD. The developing thymus is a system that is remarkably sensitive system to TCDD.



Is there an immune consequence to this level of fetal chemical exposure in the postnatal animals? We can readily show postnatal immune deficits following this kind of exposure to DES. Both cell-mediated and humorally mediated immune functions are depressed.

These animals also show more tumors if challenged with tumor cells. This is one indicator of actual immune capability. Although it's not shown here, if we challenge these mice with a pathogen we again find that they are immunosuppressed. So there are consequences to this level of early exposure.

Are there consequences in humans? That's a difficult question, but DES is one chemical where there is a human cohort and we have some information. The majority of these studies with the DES cohort are not immunotoxicity studies, there have been a few. In general I'd say they were weak studies, and I don't mean that in a critical way; there are limits to what we can do in epidemiology studies with humans.

These studies have suggested altered lymphoproliferative responses and NK-cell activity, as well as signs of increased infection. Autoimmune disease maybe is one of the more interesting ones. In 1988, Noller published his paper on the DES cohort. When all autoimmune diseases were grouped the DES cohort was found to be slightly but significantly different. There was more autoimmunity in that cohort. There have been attempts to replicate this finding in smaller studies, some suggest it's an effect and some don't.

This possibility that early chemical exposure may increase postnatal autoimmune disease is being considered now, and some limited rodent studies have been done to try to address the question. Al Silverstone at Syracuse has been exposing rodents genetically-predisposed to autoimmune disease to low levels of estrogen (DES and estradiol) during development, and has found that this will both potentiate and exacerbate an autoimmune nephritis. Male mice don't tend to show signs of the disease, and such are limited, until after the first year of life. With low-level, single-dose exposure prenatally to DES males become autoimmune at about five months of age. The females are both induced in earlier age and their responses are exacerbated from a single exposure to DES or estradiol during development.

Similar results have been obtained in autoimmune mice exposed to TCDD during development, by the very same investigator (Silverstone).

As we considered data gaps in this system, I commented that developmental immunotoxicity is a field in its infancy. There are many immunotoxicants that we know are developmental immunotoxicants.

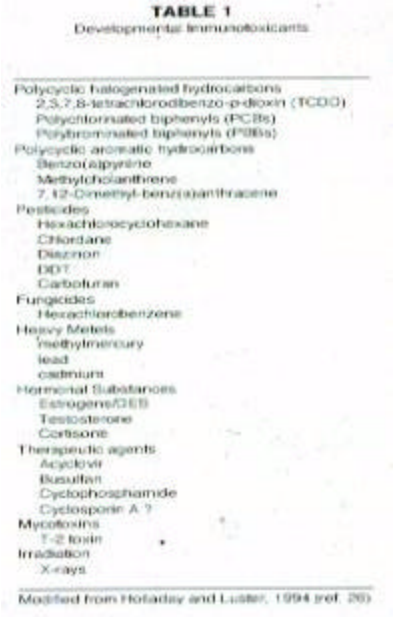


TABLE 1
Developmental Immunotoxicants

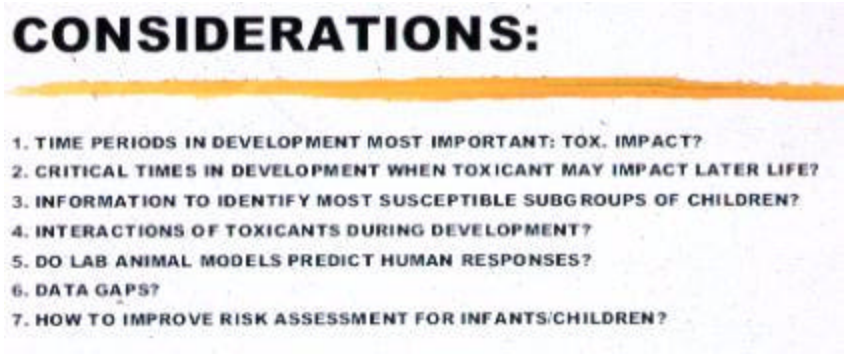
Polycyclic halogenated hydrocarbons
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)
Polychlorinated biphenyls (PCBs)
Polybrominated biphenyls (PBBs)
Polycyclic aromatic hydrocarbons
Benzo(a)pyrene
Methylcholanthrene
7,12-Dimethyl-benz(a)anthracene
Pesticides
Hexachlorocyclohexane
Chlordane
Diazinon
DDT
Carbofuran
Fungicides
Hexachlorobenzene
Heavy Metals
methylmercury
lead
cadmium
Hormonal Substances
Estrogens/DES
Testosterone
Cortisone
Therapeutic agents
Acyclovir
Busulfan
Cyclophosphamide
Cyclosporin A ?
Mycotoxins
T-2 toxin
Irradiation
X-rays

Modified from Holladay and Luster, 1994 (ref. 26)

Generally speaking, an adult immunotoxicant is a developmental immunotoxicant. The amount that crosses the placenta is critical. With some of these agents not a lot gets across the placenta.

Cyclosporin A is probably a good example of the latter. Cyclosporin A may be the drug that's most responsible for our current ability to transplant organs and maintain the transplant. Therefore, there are a number of women who have had an organ transplant and want to do what we all want to do, get back to a normal life. These women are having children, with the outcome being watched carefully. Some of the children are born leukopenic, which is a bit of a red flag. Cyclosporin A has what appears to be the ability to alter development of self-recognition in immune cells. For this reason I have some concern about this cohort, and the possibility that they might become autoimmune when puberty comes.

There are quite a number of immunotoxicants that we know have developmental effects but we don't know much more than that. The literature database needs to be expanded in that arena.



We were asked to address a set number of considerations, so I'll finish with this slide: What are the time periods in development that are most important ? What are the critical times in development when the toxicant may have its greatest effect on postnatal life? We really don't know. For most of these chemicals we really don't know.

Again, most studies have been in the latter half of gestation in the mouse model. I think it would be very important to study earlier events. Some studies of earlier events have been done with chlordane and lead, but other than this the period has not been studied.

Critical windows: We were really afraid to try to nail down any chemical, any immunotoxicant in the literature in the last children's workshop.

Information to identify most susceptible subgroups of children: Well, some of the studies using mice that are predisposed to development of autoimmune disease are going to be helpful in this regard, so there are steps in that direction.

Interaction of toxicants: This is a chemical mixtures question. This has not been studied in the developmental immunotoxicology realm. My lab has a chemical mixture RFA funded for immunotoxicity, but the exposures are all postnatal. I'm not aware that anybody that is doing this type of work in a prenatal realm, so this certainly would be a data gap.

Do lab models predict human responses? I would say, based on the data available, probably reasonably well. Our mouse model appears fairly good for predicting human responses but there are certainly many unknowns.

How to improve risk assessment? I'd like to see the National Toxicology Program's paradigm expanded into a developmental arena. I think it would be a helpful thing to do. And, again, there are certainly human cohorts – such as women taking chemotherapeutics to prevent rejection of organ grafts, allografts , or to control autoimmune disease during pregnancy, that would be excellent groups to follow postnatally. These women have been exposed to cocktails of immunosuppressants including cyclosporin A, azathioprine and so on, depending on the individual need in controlling their response. Following offspring of these people postnatally would be a very good idea.